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Negative Thyroid Transcription Factor 1 Expression Defines an Unfavorable Subgroup of Lung Adenocarcinomas

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Introduction: Thyroid transcription factor 1 (TTF1) is a master regulator of pulmonary differentiation that is downregulated in a subset of lung adenocarcinoma, of which the clinicopathologic characteristics were not fully clarified.

Methods: One thousand forty-two lung adenocarcinoma patients who underwent surgery were investigated for clinic characteristics, histologic subtyping, and spectrum of well-identified driver mutations. TTF1 expression was correlated with these clinicopathologic factors and survival.

Results: Compared with TTF1 positive (TTF1+) patients, the 133 negative individuals (12.8%, TTF1−) were more likely to be male ($p = 0.006$) and heavy smokers ($p = 0.002$) who had larger tumor size ($p < 0.001$) and more advanced disease stage ($p < 0.001$). TTF1− presented more in solid and invasive mucinous-predominant carcinomas (both $p < 0.001$), whereas TTF1+ was identified in 100% patients with adenocarcinoma in situ, minimally invasive and lepidic-predominant adenocarcinomas. The TTF1− tumors harbored the known driver mutations in significantly low frequency compared with TTF1+ adenocarcinomas (57.8% versus 78.1%, $p < 0.001$), especially in epidermal growth factor receptor (*EGFR*) mutations (37.6% versus 60.7%, $p < 0.001$). There was no significant difference in recurrence-free survival between the TTF1− and TTF1+ patients, either for the whole cohort or stratified by pathologic stage, or among the driver mutation-defined subsets. However, recurrence of multiple metastases was more likely to occur in patients with TTF1− adenocarcinomas (88.1% versus 32.4%, $p < 0.001$). Multivariate analysis revealed that TTF1− independently predicted both poor postrecurrence survival (hazard ratio = 1.664; 95% confidence interval , 1.097–2.524; $p = 0.017$) and unfavorable overall survival (hazard ratio = 1.553; 95% confidence interval , 1.013–2.381; $p = 0.043$).

Conclusions: TTF1− correlated with solid and invasive mucinous subtypes of lung adenocarcinoma and lower frequency of *EGFR* mutations. It defines a subgroup of lung adenocarcinomas with unfavorable outcomes.

Key Words: Thyroid transcription factor 1, Lung adenocarcinoma, Subtype, Driver mutation, Survival

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Thyroid transcription factor 1 (TTF1), a homeodomain-containing nuclear transcriptional protein of the *Nkx2* gene family, is a transcription factor that regulates the expression of multiple genes involved in lung development.[1] In normal lung, TTF1 plays a decisive role in the maintenance of the function of terminal respiratory unit cells.[2] Positive TTF1 (TTF1+) staining by immunohistochemistry (IHC) has been detected in primary lung adenocarcinomas and was used as a diagnostic marker.[3,4] TTF1 controls tumor differentiation and limits metastatic potential in vivo. Upregulation of TTF1 was correlated with favorable survival and downregulation linked to loss of differentiation, enhanced tumor seeding ability, and increased metastatic proclivity.[5] Moreover, a recent investigation using a genetically engineering mouse model indicated that lung adenocarcinomas with *KRAS* and *TP53* mutations required additional alterations, such as loss of TTF1 expression, to initiate the metastatic cascade.[6] Also, other major oncogenic mutations in lung adenocarcinoma have been found to occur in *EGFR, BRAF, HER2, ALK, ROS1,* and *RET*. Those gene mutations defined subsets of lung cancers that could be amenable to treatment with specific kinase inhibitors.[1] However, whether the patients with negative TTF1 (TTF1−) expression lung adenocarcinoma harbor those targetable mutations are still largely unknown.

The International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society has provided a new classification for lung adenocarcinoma, which has been reported to be associated with prognosis.[7] Previous studies have been demonstrated that TTF1+ expression was associated with lepidic, acinar, papillary, and micropapillary predominant adenocarcinoma but did not draw a definite conclusion at the association of TTF1− expression

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with this novel classification system in lung adenocarcinoma because of the limited number of cases.[8–16]

Here, we analyzed 1042 resected pulmonary adenocarcinomas for TTF1 expression and described the clinicopathologic and mutational characteristics in patients with TTF1− tumors. These investigations allowed us to define the specific characteristics associated with TTF1− lung adenocarcinomas.

PATIENTS AND METHODS

Between 2008 and 2013, consecutive patients with pulmonary tumors were prospectively included. After preoperative work-up (enhanced thoracic computed tomography, abdominal ultrasonography, brain magnetic resonance imaging, and bone scan for all patients and positron emission tomography/computed tomography for some) to exclude regional and systemic disease, patients underwent surgery with curative intent at Fudan University Shanghai Cancer Center. Lymphadenectomy was routinely done for all patients, and adjuvant chemotherapy was suggested for those who had nodal metastases. Inclusion criteria for this study included (1) patients with pulmonary tumors underwent complete resection with curative intent; (2) pathologically confirmed lung adenocarcinomas and sufficient tissue for comprehensive mutational analyses; (3) those with history of adenocarcinomas from other organs were excluded from this study. Clinical data were collected from the prospectively maintained database. Disease stage was based on the 7th edition of the American Joint Committee on Cancer staging manual.[17] Recurrence-free survival (RFS) and overall survival (OS) were recorded based on follow-up clinic or telephone. The Institutional Review Board of Fudan University Shanghai Cancer Center approved this study. All patients gave written informed consent.

Histologic Evaluation and Immunohistochemical Analysis of TTF1

IHC was performed on 4-μm thick formalin-fixed, paraffin-embedded sections. Slides were deparaffinized and pretreated with 1 mmol/liter ethylenediaminetetraacetic acid and heat-mediated antigen retrieval solution in a microwave oven. Further steps were done at room temperature in a hydrated chamber. Slides were preincubated in 20% normal goat serum. TTF1 (1:100, 8G7G3/1, DAKO, Glostrop, Denmark) were applied. The slides were then washed in Tris–HCl and detected with horseradish peroxidase-conjugated anti-rabbit EnVision+ kit (DAKO). All slides were counterstained with hematoxylin. Adenocarcinoma was further confirmed using IHC method. Solid-predominant tumors were diagnosed based on some amount (≥5%) of other histologic patterns (lepidic, acinar, papillary, or micropapillary). Two pathologists (L.S. and Y.L.) blindly reviewed the slides. We used whole tissue blocks to identify TTF1 status. Nuclear staining of tumor cells was considered TTF1+. Tumors with completely no TTF1 expression in nuclei were defined as TTF1−. All tumors were classified according to the new classification system.[7]

Mutational Analysis

EGFR (exons 18–22), *HER2* (exons 18–21), *KRAS* (exons 2–3), and *BRAF* (exons 11–15) were amplified by polymerase chain reaction (PCR) using cDNA from each tumor specimen. Sanger sequencing was then performed to analyze the amplified products. A combination strategy of quantitative real-time PCR and reverse transcriptase PCR was used to detect *ALK*, *ROS1*, and *RET* fusions, with validation using fluorescent in situ hybridization.[18–21]

Statistical Analysis

Pearson's χ^2 test or Fisher's exact test was used to investigate the associations between the categorical variables. Comparison of continuous variables was examined by independent Student's *t* test and Mann–Whitney *U* test. The survival distribution was analyzed using the Kaplan–Meier method, and log-rank tests were employed for comparisons between two categories in univariate analysis. Multivariate survival analysis was conducted using the Cox proportional hazards regression (forward likelihood ratio model) to identify independent prognostic factors. All statistical analyses were two-sided, with *p* value less than or equal to 0.05 indicative of statistical significance, and performed using SPSS (version 19.0 IBM Corporation, Armonk, NY).

RESULTS

Clinicopathologic Data and Correlation With TTF1 Expression

A total of 1042 consecutive patients with lung adenocarcinoma were included in this study, of which 133 (12.8%) cases with completely negative TTF1 expression in nuclei. Clinicopathologic parameters analyzed included age, sex, smoking history, tumor size, lymph nodal status, and pathologic stage. Compared with TTF1+ adenocarcinoma, negative patients are more likely to be male ($p = 0.006$) and heavy smokers ($p = 0.002$). They also had larger tumor size ($p < 0.001$) and more advanced disease stage ($p < 0.001$). In addition, TTF1 expression was not significantly associated with age ($p = 0.219$) and lymph node metastasis ($p = 0.753$; Table 1).

Association Between TTF1 and Histologic Subtypes

Among TTF1− group, the major subtype was acinar-predominant adenocarcinomas (36.8%), followed by solid (32.3%) and invasive-mucinous (18.1%) subtypes. Of the TTF1+ tumors, acinar (48.6%), solid (13.1%), and papillary (12.5%) were in the majority. There were no adenocarcinoma in situ, minimally invasive adenocarcinomas, or lepidic-predominant tumors in TTF1− group. However, compared with the TTF1+ adenocarcinomas, TTF1− tumors presented more as solid and invasive mucinous subtypes (both $p < 0.001$), whereas less in acinar component ($p = 0.007$). Among the 86 invasive-mucinous tumors, there were 18 (20.9%) cases with pure-mucinous component, which were all negative for the TTF1 staining. In addition, the two enteric cases showed no TTF1 staining (Table 1).

Status of Common Oncogenic Mutations

The prevalence of the driver mutations was similar as previously reported for the study population.[2,19–21] Compared with TTF1+ tumors, TTF1− adenocarcinomas harbored

TABLE 1. Clinicopathologic Factors of the Whole Cohort

Variable	TTF1− (n = 133)	TTF1+ (n = 909)	*P*
Gender (female/male)	57/76	506/403	0.006
Age (mean ± SD, yr)	60.1 ± 10.9	59.6 ± 9.9	0.219
Smoking history	61 (45.9%)	287 (31.6%)	0.002
Tumor size (mean ± SD, cm)	3.60 ± 2.13	2.63 ± 1.47	<0.001
N status			0.753
N0	81 (60.9%)	581 (63.9%)	
N1	15 (11.3%)	102 (11.2%)	
N2	37 (27.8%)	226 (24.9%)	
Pathologic stage			<0.001
0	0	15 (1.7%)	
I	58 (43.6%)	520 (57.2%)	
II	34 (25.6%)	101 (11.1%)	
III	41 (30.8%)	273 (30.0%)	
Subtypes			
AIS	0	19 (2.1%)	<0.001
MIA	0	29 (3.2%)	<0.001
Lepidic	0	105 (11.6%)	<0.001
Acinar	49 (36.8%)	442 (48.6%)	0.007
Papillary	14 (10.5%)	114 (12.5%)	0.468
Micropapillary	1 (0.8%)	19 (2.1%)	0.499
Solid	43 (32.3%)	119 (13.1%)	<0.001
Invasive mucinous	24 (18.1%)	62 (6.8%)	<0.001
Enteric	2 (1.5%)	0	<0.001
Mutational status			
EGFR mutation	50 (37.6%)	552 (60.7%)	<0.001
KRAS mutation	13 (9.7%)	67 (7.4%)	0.363
HER2 mutation	2 (1.5%)	25 (2.8%)	0.564
BRAF mutation	4 (3.0%)	9 (1.0%)	0.076
ALK fusion	5 (3.8%)	37 (4.0%)	0.836
ROS1 fusion	2 (1.5%)	7 (0.8%)	0.329
RET fusion	1 (0.8%)	12 (1.3%)	1.000
Pan-negative	56 (42.1%)	200 (22.0%)	<0.001

TTF, thyroid transcription factor; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinomas.

significantly lower frequency of *EGFR* mutations (37.6% versus 60.7%, $p < 0.001$) while inclined to have more *BRAF* mutation (3% versus 1%, $p = 0.076$). TTF1− adenocarcinomas were more likely to be "pan-negative" for the common driver mutations (42.1% versus 22.0%, $p < 0.001$; Table 1).

Survival Analysis

Survival outcomes were compared between 119 TTF1-negative patients and 332 TTF1+ patients who had been followed up for over 12 months since surgery. The baseline characteristics of the two groups were well balanced with each other (Table 2).

On univariate analysis, although the two groups seemed to share similar RFS ($p = 0.416$, Fig. 1*A*), the patients with TTF1− tumors had a worse OS than the positive cases (5-year OS: 42.8% versus 72.8%, $p = 0.001$, Fig. 1*B*). When the survival analysis was limited to stage I patients, the survival difference between the two groups was significant regarding OS ($p = 0.002$) but not for RFS ($p = 0.556$; Fig. 1*C* and *D*). When confined into each driver mutation-defined subsets, there was no OS difference between TTF1− and TTF1+ groups for *EGFR* or *KRAS*-mutated tumors ($p = 0.157$ and $p = 0.699$, respectively). However, for those with "pan-negative" mutations, TTF1− patients presented inferior OS compared with TTF1+ ones (5-year OS: 46.4% versus 77.9%, $p = 0.041$, Fig. 1*E* and *F*).

TABLE 2. Baseline Characteristics of the Patients Included for Survival Analysis

Variable	TTF1− (n = 119)	TTF1+ (n = 332)	*P*
Gender (female/male)	57/62	177/155	0.310
Age (mean ± SD, yr)	60.3 ± 11.2	58.4 ± 10.0	0.172
Surgery			1.00
Lobectomy	117 (98.3%)	325 (97.9%)	
Sublobar resection	2 (1.7%)	7 (2.1%)	
Pathologic stage			0.132
IA	30 (25.3%)	108 (32.5%)	
IB	23 (19.3%)	51 (15.4%)	
II	28 (23.5%)	53 (16.0%)	
III	38 (31.9%)	120 (36.1%)	
Lymphovascular invasion			0.279
Present	32 (26.9%)	107 (30.8%)	
Absent	87 (73.1%)	225 (69.2%)	
Adjuvant chemotherapy			0.686
Yes	67 (56.3%)	194 (58.4%)	
No	52 (43.7%)	138 (41.6%)	

TTF1, thyroid transcription factor 1.

On multivariate survival analysis adjusting for gender, age, smoking history, histologic subtype, pathologic stage, lymphovascular invasion, and *EGFR* mutational status, TTF1− proved to be an independent predictor of poor OS for patients with adenocarcinoma (hazard ratio = 1.553; 95% confidence interval , 1.013–2.318; $p = 0.043$; Table 3).

A total of 59 (49.6%) TTF1− and 171 (51.5%) TTF1+ patients experienced disease recurrence. When comparing pattern of recurrence, we found that the recurrence of multiple metastases was more likely to occur in patients with TTF1− adenocarcinomas (88.1% versus 32.4%, $p < 0.001$). As to postrecurrence survival (PRS), TTF1− patients had significantly worse PRS than TTF1+ adenocarcinoma patients on univariate analysis ($p = 0.001$, Fig. 2). Pathologic stage, TTF1 status, and EGFR-tyrosine kinase inhibitor treatment after recurrence were independent predictors of PRS as demonstrated on multivariate analysis (Table 4). In addition, there seemed no difference in OS ($p = 0.749$) or PRS ($p = 0.505$) categorized by different TTF1 status for those who have received EGFR-tyrosine kinase inhibitor therapy (Fig. 3).

DISCUSSION

TTF1, a homeodomain transcription factor also known as NKX2-1, is a reliable lineage marker for terminal

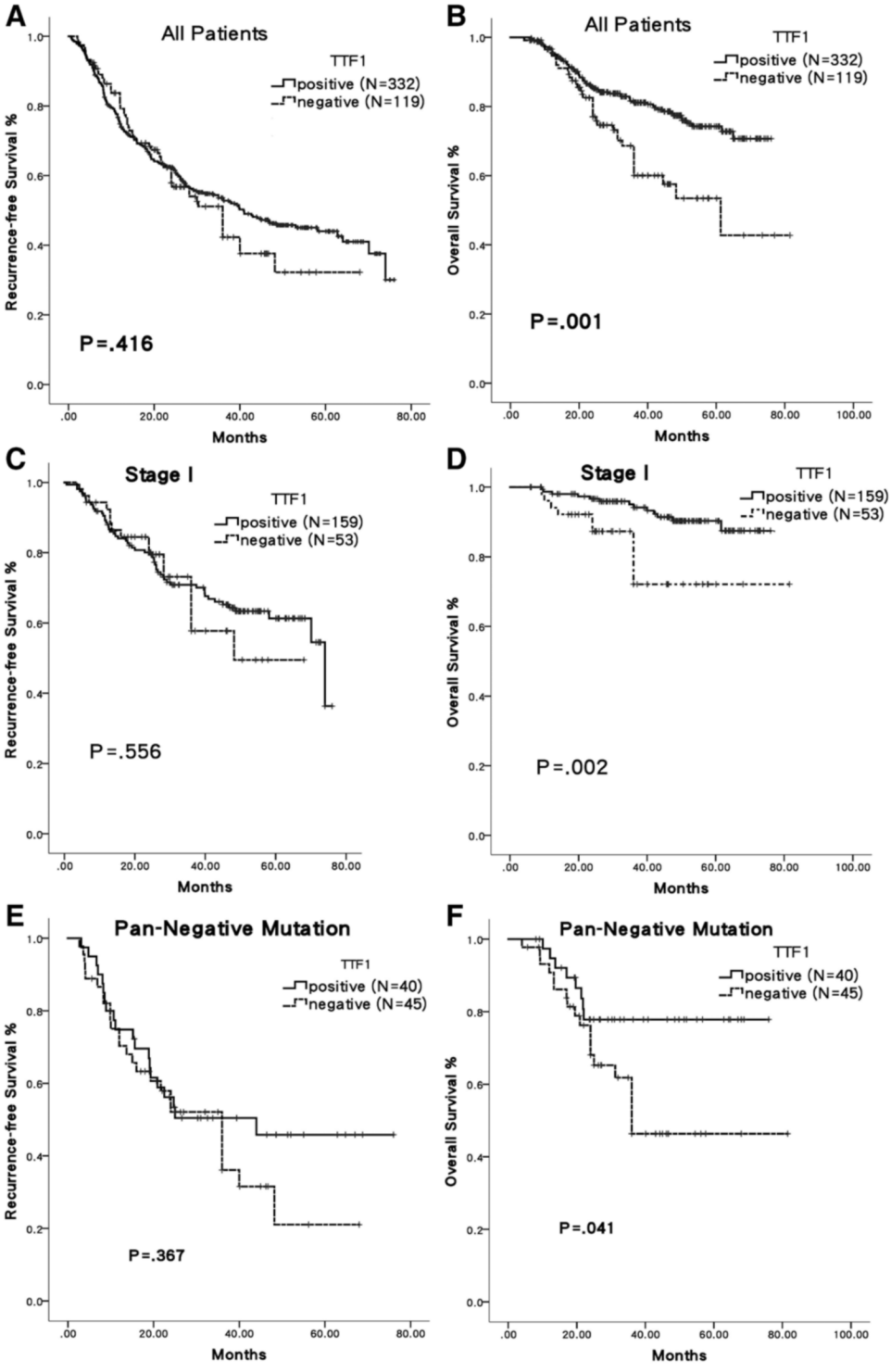

FIGURE 1. Kaplan–Meier survival curves for recurrence-free survival (RFS)and overall survival (OS) according to TTF1 status. *A*, RFS in all patients; *B*, OS in all patients; *C*, RFS in pathologic stage I patients; *D*, OS in pathologic stage I patients; *E*, RFS in the patients with tumors of "pan-negative" mutation; *F*, OS in the patients with tumors of "pan-negative" mutation.

respiratory unit cells. Mouse model researches revealed that downregulation of TTF1 would link to loss of differentiation, enhanced tumor seeding ability, and increased metastatic proclivity in lung adenocarcinomas.[5,6] In this study, we reviewed a large sample of completely resected lung adenocarcinomas to have a comprehensive assessment of the clinicopathologic characteristics, mutational status, patterns of recurrence, and survival associated with TTF1− adenocarcinoma. The results demonstrate that a lack of TTF1 expression is identified more frequently in male smokers with large tumors that present advanced disease stage. Once relapsed, patients with TTF1− adenocarcinomas inclined to experience multiple metastases. These data validate the conclusion of the basic studies from a clinical point of view.

TABLE 3. Independent Predictors of Overall Survival

	HR	95% CI	*P*
Univariate analysis			
Gender, male vs. female	1.868	1.265–2.760	0.002
Age	0.887	0.980–1.018	0.887
Smoke, never vs. ever	0.003	1.210–2.598	0.003
Subtypes			
AIS/MIA/Lepidic	—	—	<0.001
Acinar	7.064	0.971–51.41	0.054
Papillary	10.31	1.376–77.28	0.023
Micropapillary	14.09	1.274–155.7	0.031
Solid	18.41	2.529–134.0	0.004
Invasive mucinous	3.856	0.400–37.14	0.243
Enteric	13.18	0.824–210.8	0.068
Pathologic stage, I vs. II/III	0.234	0.147–0.372	<0.001
Lymphovascular invasion, absent vs. present	0.368	0.251–0.539	<0.001
EGFR mutation, no vs. yes	1.784	1.217–2.614	0.003
TTF1, negative vs. positive	1.991	1.332–2.975	0.001
Multivariate analysis			
Gender, male vs. female	1.477	0.859–2.540	0.158
Smoke, never vs. ever	1.105	0.653–1.871	0.710
Subtypes			
AIS/MIA/Lepidic	—	—	0.099
Acinar	3.745	0.506–27.725	0.196
Papillary	4.960	0.650–37.85	0.123
Micropapillary	5.087	0.449–57.69	0.189
Solid	6.592	0.874–49.73	0.067
Invasive mucinous	2.148	0.217–21.28	0.513
Enteric	11.34	0.658–195.5	0.095
Pathologic stage, I vs. II/III	0.323	0.191–0.549	<0.001
Lymphovascular invasion, absent vs. present	0.668	0.434–1.028	0.067
EGFR mutation, no vs. yes	1.233	0.802–1.895	0.339
TTF1, negative vs. positive	1.553	1.013–2.381	0.043

EGFR TKI, epidermal growth factor receptor tyrosine kinase inhibitor; TTF1, thyroid transcription factor 1; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinomas; CI, confidence interval; HR, hazard ratio.

In 2011, a new lung adenocarcinoma classification system was proposed by the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society.[7] In this study, TTF1− adenocarcinomas mostly present as acinar, solid, and invasive-mucinous subtypes but none as minimally invasive adenocarcinomas or lepidic component, which is in consistence with the previous report.[15] Moreover, all pure mucinous adenocarcinomas were negative for TTF1 staining, indicating that TTF1 insufficiency may induce mucinous adenocarcinoma of the lung.[22]

Molecularly, lung adenocarcinoma can be classified into oncogenic driver mutation-defined subsets,[2,19–21] each with potentials for targeted therapies. Our data indicated that TTF1− adenocarcinomas are negatively associated with *EGFR* mutations, which is in accordance with previous

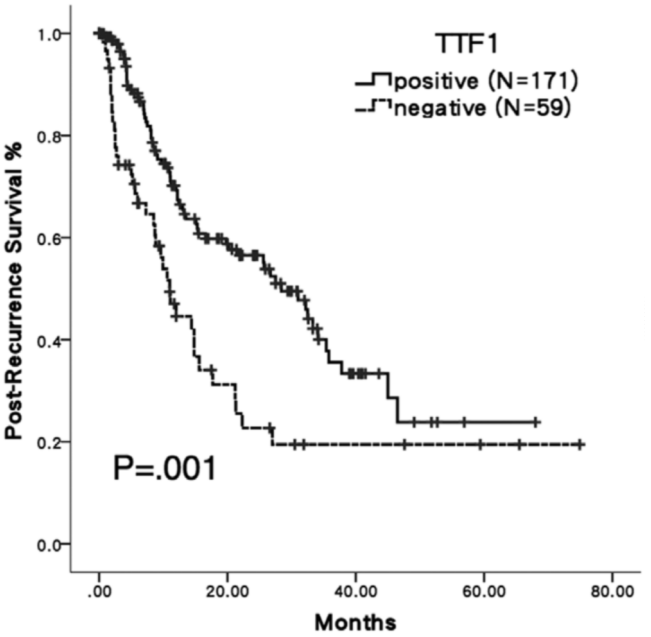

FIGURE 2. Kaplan–Meier survival curve for postrecurrence survival.

TABLE 4. Independent Predictors of Postrecurrence Survival

	HR	95% CI	*P*
Univariate analysis			
Gender, male vs. female	1.425	0.964–2.109	0.076
Age	1.009	0.990–1.029	0.348
Smoke, never vs. ever	0.815	0.555–1.196	0.296
Subtypes			
Lepidic	—	—	0.080
Acinar	2.883	0.395–21.02	0.296
Papillary	3.674	0.490–27.56	0.206
Micro-papillary	2.470	0.223–27.35	0.461
Solid	5.046	0.693–36.76	0.110
Invasive mucinous	1.793	0.186–17.25	0.613
Pathologic stage, I vs. II/III	0.620	0.390–0.985	0.043
Lymphovascular invasion, absent vs. present	0.830	0.566–1.218	0.342
EGFR mutation, absent vs. present	1.517	1.034–2.225	0.033
TTF1, negative vs. positive	2.026	1.356–3.025	0.001
Adjuvant chemotherapy, no vs. yes	1.197	0.748–1.917	0.454
Adjuvant radiotherapy, no vs. yes	1.300	0.852–1.982	0.224
EGFR TKIs, no vs. yes	1.840	1.155–2.931	0.010
Multivariate analysis			
Pathologic stage, I vs. II/III	0.536	0.332–0.865	0.011
EGFR mutation, absent vs. present	1.244	0.832–1.861	0.287
TTF1, negative vs. positive	1.664	1.097–2.524	0.017
EGFR TKIs, no vs. yes	1.887	1.151–3.093	0.012

EGFR TKI, epidermal growth factor receptor tyrosine kinase inhibitor;TTF1, thyroid transcription factor 1; CI, confidence interval.

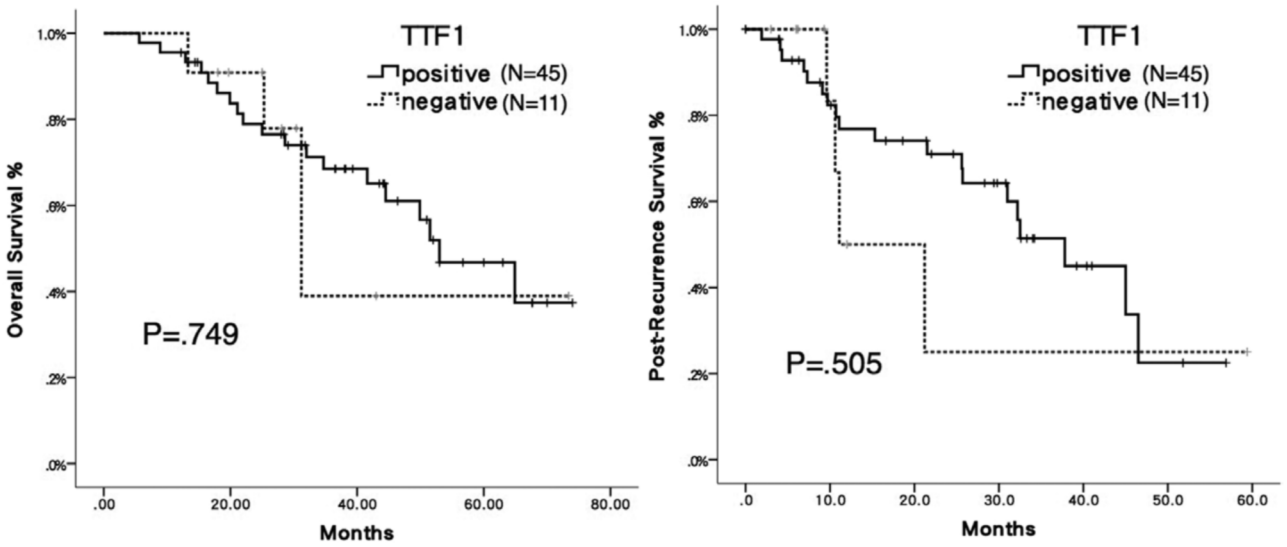

FIGURE 3. There was no significant difference in overall survival (OS; $p = 0.749$) or postrecurrence survival (PRS; $p = 0.505$) categorized by different thyroid transcription factor 1 (TTF1) status for those who have received epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) therapy.

studies.[16,23] Although they have a higher proportion of *BRAF* mutation (3%), 42.2% of this group could not be defined by any of the seven common driver mutations, about twice the ratio of TTF1+ adenocarcinoma. More attention should be paid to this aggressive lung adenocarcinoma subtype to identify new molecular targets.

The association of TTF1 and survival in lung cancer patients has been investigated in numerous studies, most of which have reported the poor prognostic role of negative TTF1 expression in lung adenocarcinoma.[9–15,24–29] Here, we further revealed that this subset was an independent poor prognostic marker for OS validated by multivariate survival analysis incorporating clinicopathologic variables and status of well-identified driver mutations. However, when confined into the common oncogenic mutated tumors, there was no OS difference between the TTF1− and TTF1+ groups (*EGFR*, $p = 0.157$ and *KRAS* $p = 0.699$). Contrarily, for those with "pan-negative" mutations, TTF1− patients presented inferior OS compared with TTF1+ ones ($p = 0.041$), implying the molecular mechanism for the poor prognosis of TTF1− adenocarcinomas may be associated with some unknown underpinnings but not with these driver mutations. In addition, we found that the recurrence of multiple metastases was more likely to occur in patients with TTF1− adenocarcinomas ($p < 0.001$). This aggressive pattern of recurrence provides clinical evidence for the findings in the mouse model research[6] and may explain the reason why TTF1− adenocarcinoma patients shared similar RFS but ended in much poor OS.

TTF1− defines a subgroup of lung adenocarcinomas with unfavorable outcomes, which may because of the aggressive pattern of recurrence. Future studies are warranted to investigate molecular mechanisms of the poor prognosis and identify novel therapeutic targets to benefit more patients with this aggressive disease.

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